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Lysis of Erythrocytes from Stored Human Blood by Phospholipase C (*Bacillus cereus*)

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The ability of phospholipase C (*Bacillus cereus*) to lyse erythrocytes from human blood that had been stored under Transfusion Service conditions for up to 16 weeks has been examined. When incubated at 20°C with enzyme (0.03 mg/ml, 55 units/ml) for up to 1 h fresh erythrocytes were not lysed. After about 4 weeks of storage a population of very readily lysed erythrocytes appeared. The morphological changes in erythrocytes from blood stored up to 16 weeks were examined by scanning electron microscopy. The proportion of very readily lysed erythrocytes correlated well with the proportion of spheroechinocytes I. This morphological form was shown to be preferentially removed by phospholipase C and before lysis a transient appearance of smooth spheres occurred. The decrease in blood ATP concentrations on storage was measured and found to correlate with the disappearance of discoid erythrocyte forms, but not directly with the increased susceptibility of the erythrocytes to lysis by the enzyme. However, erythrocytes of up to at least 15 weeks of age could be made less susceptible to lysis by preincubation in a medium designed to cause intracellular regeneration of ATP. During the lysis of spheroechinocytes I by electrophoretically pure recrystallized phospholipase C a rapid degradation of phosphatidylcholine, phosphatidylethanolamine and (phosphatidylserine + phosphatidylinositol) occurred together with a slower degradation of sphingomyelin.

During the storage of human blood for transfusion, erythrocytes undergo a range of alterations that include morphological and metabolic changes (Nakao *et al.*, 1959; Longster *et al.*, 1972; Rumsby *et al.*, 1974; Lichtman *et al.*, 1974; Leavitt, 1971), membrane reorganization (Westerman *et al.*, 1963; Van Gastel *et al.*, 1965; Winterbourn & Batt, 1970; Shukla *et al.*, 1978*b*) and loss of membrane components (Haradin *et al.*, 1969; Weed *et al.*, 1974; Rumsby *et al.*, 1977). During the aging process, the erythrocyte membrane becomes more susceptible to attack by sublytic amounts of phospholipase C (Shukla *et al.*, 1978*b*).

We have examined the effect of storage of human blood on the susceptibility of erythrocytes to lysis by phospholipase C (*Bacillus cereus*) and looked for correlations between this and erythrocyte morphology and blood ATP concentrations.

Materials and Methods

Storage of blood

Human blood (group O, Rh^+) in acid/citrate/dextrose (blood/anticoagulant, 6:1, v/v) was obtained in plastic donation bags from the Leeds Regional Blood Transfusion Centre within 1 day of donation. Blood was stored at 4°C and samples were withdrawn aseptically from the donation bag as required after thorough mixing. No bacterial contamination was detected when any of the blood samples used in this work was spread on agar plates and incubated at 37°C for 20 h. Erythrocytes were separated from whole blood by centrifugation at 2000*g* for 6 min and were washed by resuspending three times in 0.9% NaCl to a 50% (v/v) dilution of packed cells.

Scanning electron microscopy

Washed erythrocytes either with or without phospholipase C treatment were incubated in 1% glutaraldehyde in Spinner's salt solution (Bessis & Weed, 1972) (1 vol. of cell suspension/100 vol. of glutaraldehyde solution) at room temperature for 30 min. The post-fixation procedure used was that described by Bessis & Weed (1972), except that the alcohol concentrations for the progressive dehydration were 20, 50, 80 and 100%. The samples were finally coated with gold in an argon atmosphere and examined with a Cambridge Stereoscan 600 electron microscope.

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Regeneration of erythrocyte ATP

Cells were washed twice in 0.9% saline and then resuspended to the original haematocrit value in a medium comprising inosine (5.3 g/litre), adenine (0.27 g/litre), NaCl (0.11 M) and 82.5 mOSM-sodium phosphate buffer (pH 7.4). After 2 h incubation at 37°C the cells were washed twice with 0.9% NaCl and then resuspended to 50% dilution of packed cells in 0.9% NaCl.

Extraction and analysis of phospholipids

Portions (10 ml) of suspended erythrocytes were pipetted in duplicate into methanol (70 ml) with mixing directly into homogenization vessels on a top-drive homogenizer. Chloroform (10 ml) was added and the cells were homogenized. The extract was filtered through pre-washed glass-fibre filter paper. The residue was then re-extracted with 70 ml of chloroform/methanol (1:1, v/v) and then with 70 ml of chloroform/methanol (2:1, v/v). Lipid extracts were combined and the solvent was removed. Lipid was redissolved in 80 ml of chloroform/methanol (2:1, v/v) and partitioned against 16 ml of 0.1 M-KCl. The lower chloroform phase was recovered and the solvent was removed. Lipid was redissolved in chloroform, filtered and transferred to weighed vials. Solvent was removed and the vials plus residue were dried overnight in a desiccator over silica gel, weighed and the vial contents were dissolved in known volumes of chloroform. Individual phospholipid classes were then separated by one-dimensional t.l.c. on silica gel H with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as developing solvent. Phospholipids were made visible with I_2 vapour and identified by reference to standards chromatographed simultaneously. The chromatographic system did not separate phosphatidylserine from phosphatidylinositol. After development and identification, the phospholipid spots were scraped from the plate and the phosphorus content was determined (Bartlett, 1959).

Haemolysis conditions

Washed erythrocytes at 50% dilution (v/v) in 0.9% NaCl containing 5 mM-Tris/HCl buffer (pH 7.4) and 0.05 mM-Zn^{2+} were preincubated for 15 min on a water bath at 20°C to permit thermal equilibration. Phospholipase C to a final concentration of 55 units/ml (0.03 mg/ml) was then added. The mixture was then occasionally shaken. Portions (1 ml) were removed at various times, centrifuged (2000 *g* for 2 min) and the supernatant was removed. The extent of haemolysis was estimated by measuring the absorbance of the supernatant at 412 nm after appropriate dilution in 0.9% NaCl (*a*) and expressed as a percentage of the absorbance at 412 nm of a saponin-lysed control sample of erythrocytes (*b*). Haemolysis values have been corrected for changes in the extracellular volume arising from cell lysis by the equation:

$$\text{haemolysis (\%)} = \frac{a}{2b-a} \times 100$$

During the centrifugation, washing and incubation of erythrocytes, plastic vessels and plastic pipettes were used throughout.

Phospholipase C

Phospholipase C was isolated from the culture supernatant of *B. cereus* as described by Little *et al.* (1975) and in some cases subsequently crystallized from $(NH_4)_2SO_4$ solution (Hough *et al.*, 1978). Enzyme samples used appeared homogeneous when up to 0.105 mg of enzyme protein was analysed in the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis system of Laemmli (1970) either with or without the 2-mercaptoethanol pretreatment. The specific activity of the enzyme used was 1850 units/mg. One unit of enzyme activity releases 1 μmol of H^+ from crude egg-yolk substrate when assayed as described previously (Little *et al.*, 1975).

ATP concentrations in blood were assayed with test kits (Boehringer no. 15979; Boehringer Corp., Mannheim, Germany; and Sigma no. 366-UV; Sigma, St. Louis, MO, U.S.A.).

Results

Lysis of erythrocytes by phospholipase C

Erythrocytes from blood stored at 4°C for different periods up to 16 weeks were incubated at 20°C with phospholipase C and the time course of cell lysis was studied. Under the experimental conditions used, erythrocytes from blood of up to about 4 weeks of age were not lysed to any significant extent (< 2%). After 6 or more weeks of storage significant lysis was noted during 60 min incubation of erythrocytes with the enzyme (Fig. 1). Except for cells stored for around 15 weeks, erythrocyte lysis followed a rather unusual time course. After a lag phase of a few minutes, a fairly rapid lysis occurred, which ceased after about 40–50 min, whereupon lysis continued but at a markedly decreased rate. The extent of the rapid phase of lysis increased with the age of the erythrocytes so that at 15 weeks nearly all of the erythrocytes appeared to be lysed during the rapid phase. The general trends of increased lytic susceptibility with storage together with the development of a rapid lytic phase, the extent of which varied from effectively zero at 3 weeks to near 100% at 15 weeks were observed with every donation of blood examined (approx. 10 different donations), although individual variations were noted. The results in Fig. 1 are those obtained with one typical donation. Similar results were

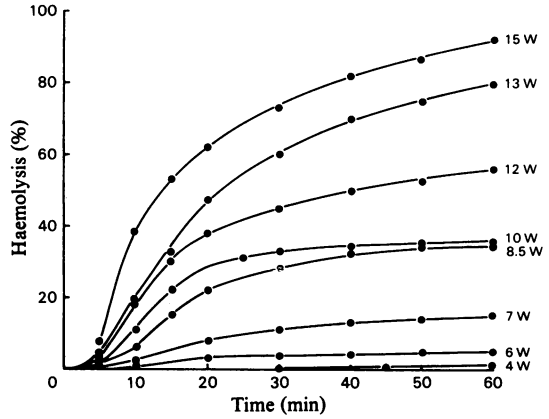


Fig. 1. *Haemolysis by phospholipase C at 20°C of stored erythrocytes*

After different periods of storage, erythrocytes were isolated from blood and incubated at pH 7.4 and 20°C with 55 units of phospholipase C/ml. Portions were removed from the incubation mixture at different times and the extent of haemolysis was measured. In the Figure, W indicates weeks of storage of the erythrocytes.

obtained with crystallized and non-crystallized enzyme and with enzyme that had been unfolded in 4 M-guanidinium chloride and then refolded by dialysis against a Zn^{2+}-containing buffer (Little & Johansen, 1979). The haemolytic activity of the enzyme was abolished when the structural zinc atoms were replaced by copper atoms, a process that causes total enzyme inactivation (Little & Otnaess, 1975) (results not shown).

Morphological changes in erythrocytes during storage for 16 weeks

The morphology of erythrocytes from blood stored up to 16 weeks was examined by scanning electron microscopy. Erythrocytes were divided into four different morphological groups: discocytes + echinocytes I; echinocytes II; echinocytes III; spheroechinocytes I. During the first few days of storage discocytes + echinocytes I predominated (Plate 1*a*). At 4 weeks, echinocytes I, II and III together with a few spheroechinocytes I were found (Plate 1*b*). At 10 weeks the major cell type was the spheroechinocyte I, with echinocytes II and III

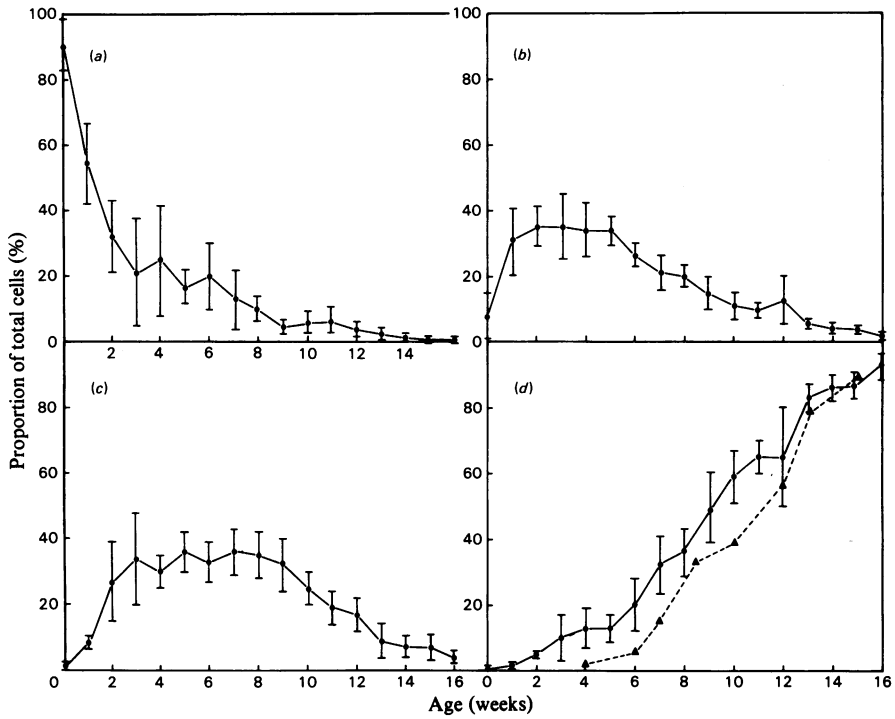


Fig. 2. *The morphological composition of erythrocytes isolated from blood after different periods of storage*

Erythrocyte morphology was studied by using scanning electron micrographs; 300–400 cells were studied/donation per week and classified by the method of Bessis (1972). For a given week four to five different donations were studied and the average results were plotted. The bar lines indicate the average ± S.D. (*a*) Discocytes + echinocytes I; (*b*) echinocytes II; (*c*) echinocytes III; (*d*) spheroechinocytes I. The percentage cell lysis after incubation of erythrocytes with 55 units of phospholipase C/ml at 20°C and pH 7.4 for 1 h (▲) is given in (*d*) and was obtained from the results in Fig. 1.

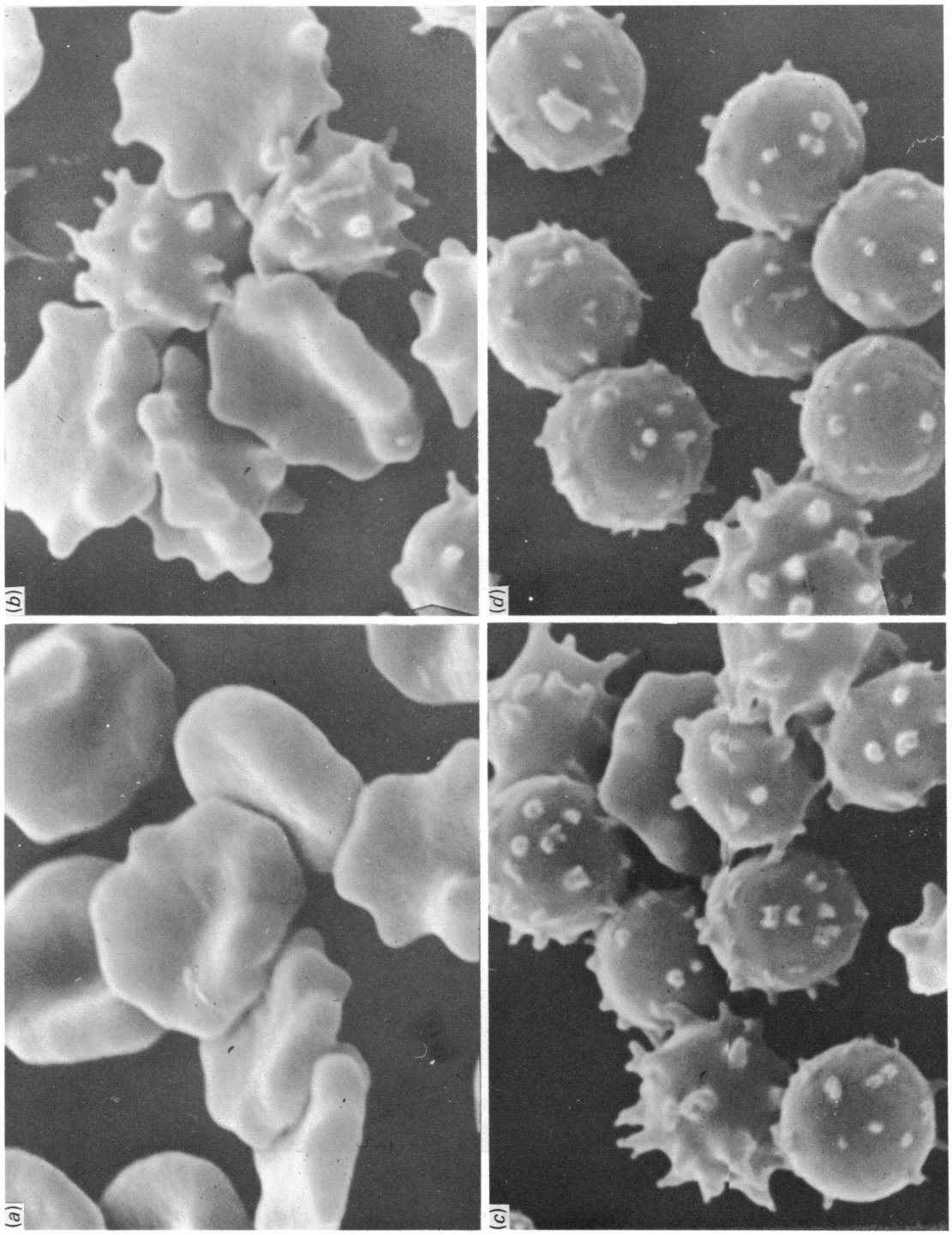
making up about 30% of the erythrocytes (Plate 1*c*), whereas at 16 weeks, almost all residual cells were spheroechinocytes I. No genuine smooth spheres were found in untreated blood of any age up to 16 weeks. At 16 weeks, about 10% haemolysis was noted in untreated whole blood in several donations and studies were not extended beyond this time. More details of these morphological changes are given in Fig. 2. Discocytes and echinocytes I have been classified together. It was found somewhat difficult to distinguish accurately between these two classes and the discocyte–echinocyte I transfor-

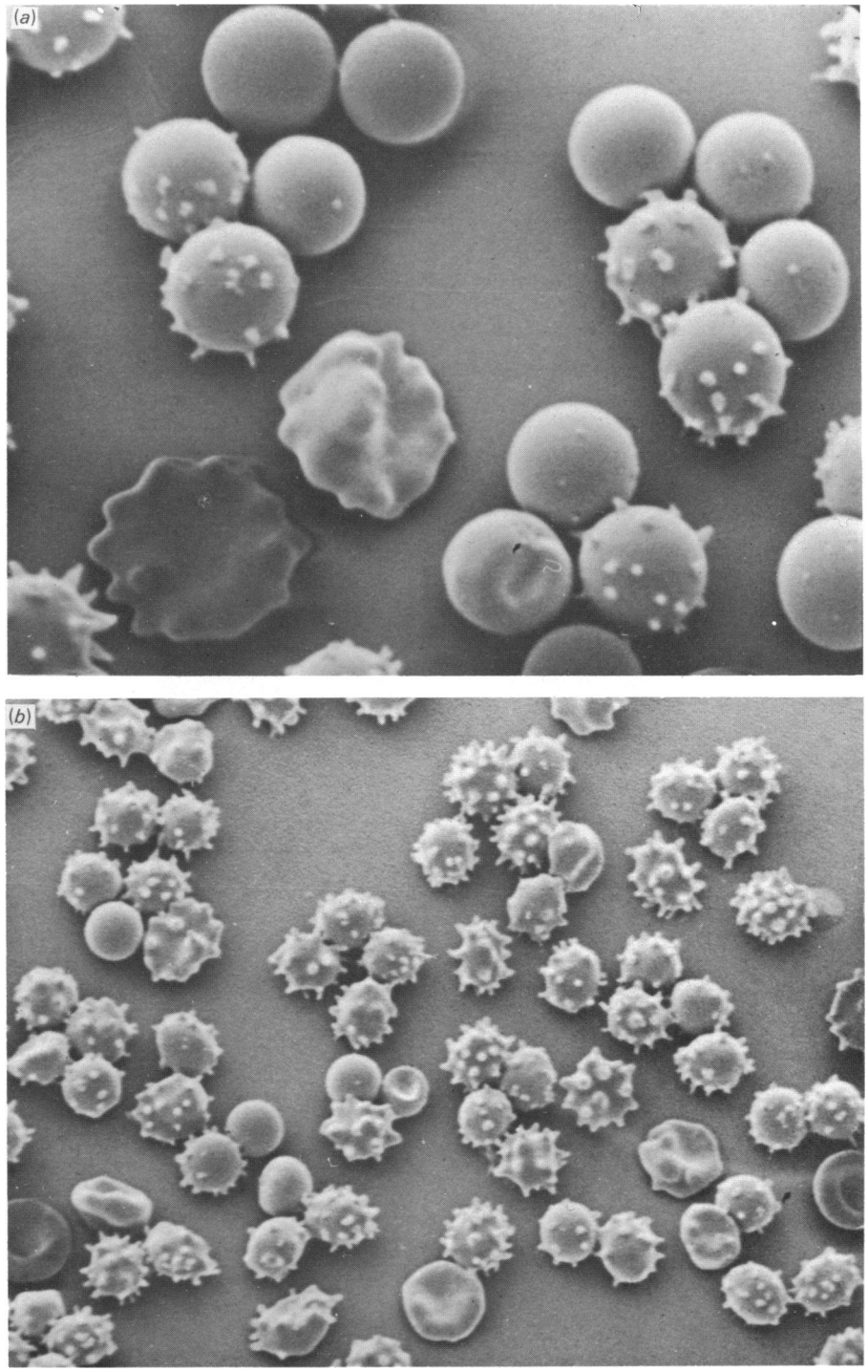
Table 1. *Effect of phospholipase C treatment on the morphological composition of stored erythrocytes*
Washed cells from blood after different periods of storage were incubated at 20°C in 0.9% NaCl/5 mM-Tris/HCl (pH 7.4)/0.05 mM-Zn^{2+} with 55 units of phospholipase C/ml. No enzyme was added to control samples. Portions were removed from the incubation mixture at different times for measurement of the extent of haemolysis and for fixation for electron microscopy (see the Materials and Methods section). Erythrocyte morphology was studied by using scanning electron micrographs of 300–400 cells/sample. The identification of morphological forms was based on the photographs of Bessis (1972).

		Cell type (% of all residual cells)						
Age of erythrocytes	Time with enzyme	Discocytes + echinocytes I	Echinocytes II	Echinocytes III	Spherо-echinocytes I	Smooth spheres	Stomato-cytes	Haemolysis (%)
4 weeks	Control (30 min)	55	12	25	8	0	0	0
	30 min	65	17	14	4	0	0	3
	60 min	62	15	20	2.5	0	0	5
	100 min	61	18	21	0	0	0	6
7 weeks	Control (15 min)	28	24	28	20	0	0	0
	15 min	31	25	26	12	5	1	7
	30 min	46	18	28	4	1	4	11
	60 min	35	19	40	2	0	4	14
10 weeks	Control (30 min)	10	6	18	66	0	0	0
	10 min	17	12	32	28	11	2	5
	20 min	23	15	25	27	8	2	22
	30 min	28	15	30	19	7	1	31
	60 min	28	21	37	9	4	1	39
13 weeks	Control (10 min)	7	4	2	87	0	0	0
	10 min	12	8	11	46	20	3	15
	15 min	16	7	15	48	10	4	35
	20 min	14	8	23	47	5	3	43
	40 min	15	14	42	26	1	2	56
	60 min	17	20	38	23	2	2	64
	90 min	16	20	54	6	2	2	72
16 weeks	Control (2 min)	1	1	6	92	0	0	0
	1 min	2	4	6	66	16	6	—
	2 min	4	2	8	38	43	5	4
	3 min	4	4	8	34	45	5	—
	4 min	5	9	16	37	29	4	18
	5 min	5	6	15	55	15	4	—
	6 min	6	11	13	54	12	4	51
	10 min	12	16	17	32	7	6	70

EXPLANATION OF PLATE 1

Scanning electron micrographs of erythrocytes isolated from blood after different periods of storage
(*a*) 1 week-old cells; (*b*) 4 week-old cells; (*c*) 10 week-old cells; (*d*) 16 week-old cells. The magnification factor is 5200.


(b)
(d)
(a)
(c)



EXPLANATION OF PLATE 2

Scanning electron micrographs of erythrocytes from 16 *week-old blood after incubation with phospholipase C* (55 *units/ml*) *at* 20°*C and pH* 7.4

(*a*) 3 min with enzyme; (*b*) 10 min with enzyme. The magnification factors are 3100 in (*a*) and 1400 in (*b*).

mation clearly occurs well before the major changes in the lytic susceptibility of the erythrocytes. One noteworthy observation concerning erythrocyte morphology and susceptibility to lysis by phospholipase C is that the proportion of spheroechinocytes I in a population of erythrocytes correlates well with the extent of the rapid phase of lysis (Fig. 2*d*).

Effect of phospholipase C treatment on erythrocyte morphology

Erythrocytes from donations of blood of different ages were incubated with phospholipase C. Samples were removed at different times and were fixed directly in glutaraldehyde and subsequently examined by scanning electron microscopy (Table 1). Phospholipase C was found to be totally inactivated within 1 min when exposed to the glutaraldehyde fixative. With erythrocytes from blood of 4, 7, 10 and 16 weeks of age, it is clear that the spheroechinocyte I is the cell type most readily removed by the enzyme. With cells of 4 and 7 weeks, the extent of haemolysis seems to follow the disappearance of this cell type. With older erythrocytes, smooth spheres and a few cells, which we have classified as stomatocytes, were detected after incubation with the enzyme. This is most apparent in an experiment with 16 week erythrocytes (Plate 2*a*), where the transient appearance of these two new cell forms can be seen. The results in Table 1 show that with 16 week cells the spheroechinocyte I content decreased from 92% of all cells to 34–38% before haemolysis had reached 10%. In addition, smooth spheres are formed during this time and their population increased to 43–45% of all erythrocytes (Plate 2*a*; Table 1). Rapid haemolysis seemed then to occur and the proportion of smooth spheres decreased markedly. During haemolysis the proportion of echinocytes, I, II and III in the intact cells increased markedly suggesting that these cell types are much more resistant to lysis or morphological changes induced by the enzyme. The general trends shown in Table 1 were reproduced with three different batches of erythrocytes.

ATP concentrations in stored blood

The ATP content of blood was measured and large decreases were noted during storage. After 12 weeks of storage, ATP concentrations were about 10% of those in fresh blood (Fig. 3). When blood ATP concentrations were compared with the changes in morphological composition of the erythrocytes, a fairly good correlation was found between the relative ATP concentration of blood and the proportion of discoid erythrocyte forms (i.e. discocytes + echinocytes I and II) in the blood (Fig. 3). No direct correlation could be found between decreased blood ATP concentrations and the increased susceptibility of erythrocytes to lysis by the enzyme.

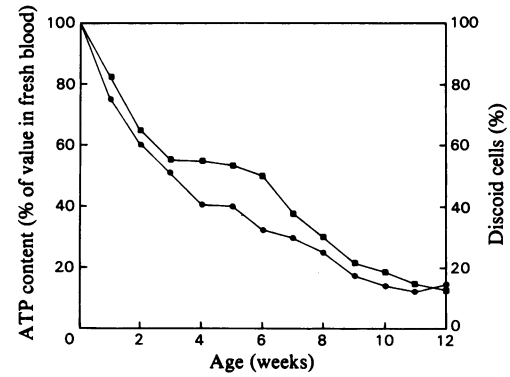


Fig. 3. *ATP concentrated in stored blood and erythrocyte morphology*

The ATP content of blood stored for different periods of time was measured and expressed as a percentage of the ATP content of freshly received blood (●), which was found to be 13.7 mg/100 ml of blood. Erythrocyte morphology was studied by using scanning electron microscopy (see the Materials and Methods section). The percentage of cells in a discoid form (i.e. discocytes + echinocytes I and II) was calculated after each week of storage of blood (■). The results are averages obtained from three different donations.

The susceptibility of erythrocytes from stored blood to lysis by phospholipase C was also examined after the erythrocytes had been preincubated in a medium designed to cause intracellular ATP regeneration (Fig. 4). By comparing the data in Figs. 1 and 4 which were obtained with erythrocytes from the same donation, it can be seen that this 'rejuvenation' treatment decreased the extent of the rapid phase of lysis of aged erythrocytes. This applied even to 15 week-old cells. However, with erythrocytes of more than about 6 weeks of age the initial rate of enzyme-induced lysis was significantly greater with cells exposed to the rejuvenation pretreatment than with the untreated cells (results not shown). Neither extending the rejuvenation treatment to 3 h nor adding glucose (0.5%, w/v) to the rejuvenation medium caused any further protection against lysis.

Phospholipid degradation during the lysis of spheroechinocytes I by phospholipase C

The loss of total phospholipid and of the individual classes of phospholipid during the lysis by the enzyme of erythrocytes from 15 week-old blood was investigated. The cells were incubated with enzyme, the lipids extracted and analysed at different times

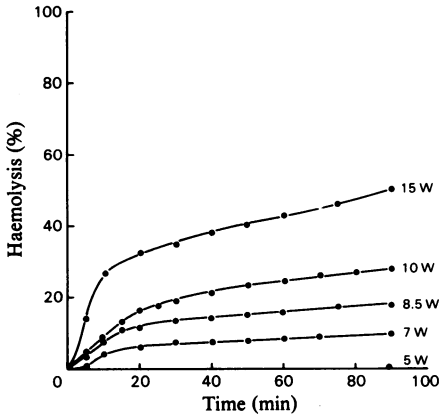


Fig. 4. *Haemolysis by phospholipase C at 20°C of stored erythrocytes after rejuvenation treatment*

After different periods of storage, erythrocytes were isolated from blood and suspended at a 50% dilution of packed cells in a rejuvenating medium designed to restore intracellular ATP concentrations. After 2h incubation at 37°C, the cells were washed and incubated at 20°C with 55 units of phospholipase C/ml. The extent of haemolysis was measured after different periods of incubation with the enzyme. Further details are given in the Materials and Methods section. In the Figure, W indicates weeks of storage of the erythrocytes.

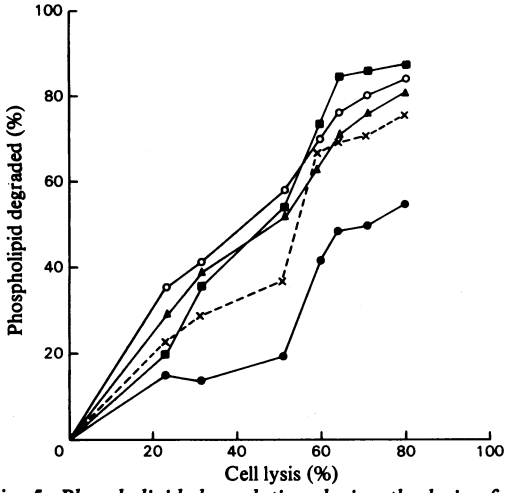


Fig. 5. *Phospholipid degradation during the lysis of 15 week-old erythrocytes*

Erythrocytes were treated with enzyme as described in the Materials and Methods section except that recrystallized enzyme at concentrations of 0.002 and 0.01 mg/ml were used. Incubation times were from 15 to 90 min. The extent of cell lysis was then measured and extractions and phospholipid analyses were carried out in duplicate. Results are expressed as percentages of untreated controls, the latter being made in quintuplicate. Symbols: O, phosphatidylcholine; ■, phosphatidylethanolamine; ▲, phosphatidylinositol + phosphatidylserine; ●, sphingomyelin; ×, total phospholipid.

and the extent of cell lysis was measured. The extraction solvents caused a very rapid and total inactivation of the enzyme. Cell lysis was accompanied by extensive degradation of phosphatidylcholine, phosphatidylethanolamine and (phosphatidylserine + phosphatidylinositol). Sphingomyelin was also degraded but at a lower rate (Fig. 5). The experiment was repeated in the presence of 5 mM-Ca^{2+}, a very powerful inhibitor of the sphingomyelinase C of *B. cereus* (Ikezawa *et al.*, 1978) and no effect on the extents of degradation of any of the phospholipids including sphingomyelin was found (results not shown).

Discussion

Fresh human erythrocytes are very resistant both to lysis and to phospholipid degradation by phospholipase C (*B. cereus*) (Roelofsen *et al.*, 1971; Zwaal *et al.*, 1971; Little *et al.*, 1975; Shukla *et al.*, 1978*b*). Shukla *et al.* (1978*b*) have shown that after about 2 weeks of storage when the erythrocytes have become echinocytic, the membrane phospholipids become more readily hydrolysed by this enzyme. The present work demonstrates that these early morphological forms are extremely resistant to lysis by phospholipase C. Periods of storage greater than 3–4 weeks gradually render an increasing proportion of erythrocytes very susceptible to lysis by the enzyme. The morphological studies suggest that the very readily lysed erythrocytes are in the spheroechinocyte I morphological form and that before being lysed they are converted into smooth spheres. A few stomatocytic cells were also formed during enzyme attack. Stomatocytic forms have also been noted during the treatment of erythrocytes with other phospholipases (Allan *et al.*, 1975; Wilbers *et al.*, 1979). Relatively high amounts of enzyme (0.03 mg/ml) were needed for lysis. The possibility that the lysis is a membrane perturbation effect arising from the high enzyme protein concentrations is ruled out by the fact that the inactive copper-substituted enzyme was non-lytic at 0.03 mg/ml. The exact nature of the membrane changes involved in the formation of the spheroechinocyte I, which render this cell type so sensitive to enzyme-induced lysis, are not clear. The mechanism might involve diacylglycerol, which is known to build up in the membrane of aging erythrocytes and may induce changes in membrane organization (Allan & Michell, 1975; Allan *et al.*, 1976, 1978; Rumsby *et al.*, 1977). It is perhaps relevant that the introduction of oleate or certain detergents into the membrane of fresh human erythrocytes renders them extremely susceptible to lysis by phospholipase C (*B. cereus*) (Roelofsen *et al.*, 1971; Rumsby *et al.*, 1979). During the storage of blood, erythrocytes lose membrane lipid that can be recovered in the form of

microvesicles (Rumsby *et al.*, 1977). For glass-stored blood, the shedding of microvesicles becomes detectable at about 2 weeks and increases during 2–8 weeks of storage. During this period the spheroechinocyte I population in glass-stored blood also increases markedly (C. Little & M. G. Rumsby, unpublished work). It therefore seems possible that the membrane changes that result in the shedding of microvesicles may also be those that render the cells susceptible to lysis by the enzyme.

The morphological changes in erythrocytes during the storage of blood follow the general trends reported by others (Longster *et al.*, 1972; Rumsby *et al.*, 1974; Shukla *et al.*, 1978*b*) with two exceptions. We found no true smooth spheres in any donation of untreated blood during 16 weeks of storage. Other workers (e.g. Longster *et al.*, 1972) appear to have classified the lightly crenated spheres as smooth spheres, whereas we have classified them as spheroechinocytes I on the basis of the photographs of Bessis (1972). In addition, the echinocyte–spheroechinocyte transition appears to have occurred rather slowly in the present work. The present data are based on a large number of donations (22) and standard deviations are indicated. The discrepancy with other workers may relate to the nature of the vessel in which the blood is stored. We have used the standard plastic transfusion bags, but have noticed repeatedly that blood from the same donation ages more rapidly in terms of erythrocyte morphology and lytic susceptibility when stored in glass bottles rather than in the plastic bags (C. Little & M. G. Rumsby, unpublished work).

One of the well-established biochemical changes occurring during the storage of blood is a decrease in ATP concentrations (Haradin *et al.*, 1969; Nakao, 1974; Feo & Mohandas, 1977). In the present work, major decreases in blood ATP concentrations were found to occur well before marked increases in the susceptibility of erythrocytes to lysis by the enzyme and no simple correlation was found between these two changes. In agreement with the report of Haradin *et al.* (1969) the decrease in ATP concentrations on storage of blood correlated well with the decrease in the proportion of discoid erythrocyte forms. This would be consistent with a key role for ATP in the disc–sphere shape transformation of erythrocytes, possibly involving an effect *via* erythrocyte phosphatase and the phosphorylation state of spectrin (Graham *et al.*, 1976; Birchmeier & Singer, 1977; Shohet & Greenquist, 1977). During the aging of blood the erythrocyte goes through a series of shape changes: discocyte → echinocyte I → echinocyte II → echinocyte III → spheroechinocyte I. In this transition, the first spheroidal cell type formed, the echinocyte III, is very resistant to lysis by the enzyme. Since phospholipase C selectively lyses the second spheroidal cell type formed (the spheroechinocyte I), no direct correlation would then be expected between decreased blood ATP concentrations and increased susceptibility to haemolysis by the enzyme. However, ATP concentrations are relevant to the susceptibility of erythrocytes to attack by phospholipase C; the replenishment of depleted ATP concentrations seems to protect both aged erythrocytes and erythrocyte 'ghosts' from attack by this enzyme (Shukla *et al.*, 1978*a*,*b*). In addition, the incubation of aged erythrocytes in a medium designed to increase intracellular ATP resulted in an overall decrease in the lytic susceptibility of the cells. The rapid phase of lysis was diminished, but not abolished. Extending the rejuvenation treatment had no additional protective effect, suggesting that some aged erythrocytes had lost the ability to respond to the treatment.

During the lysis of 15 week old erythrocytes (i.e. essentially spheroechinocytes I) by phospholipase C, phosphatidylcholine, phosphatidylethanolamine and (phosphatidylserine + phosphatidylinositol) were degraded to about equal extents, with sphingomyelin being degraded somewhat less readily. Similar results were obtained by Shukla *et al.* (1978*b*) when sublytic amounts of this enzyme were incubated with erythrocytes from 9 week-old blood.

Sphingomyelin is not normally considered to be a substrate for pure phospholipase C (*B. cereus*) (Zwaal *et al.*, 1971; Otnaess *et al.*, 1977; Roberts *et al.*, 1978) and the situation is complicated by the presence of a sphingomyelinase C in the culture filtrate of *B. cereus* (Ikezawa *et al.*, 1978). However, we find that electrophoretically pure recrystallized phospholipase C (*B. cereus*) degrades sphingomyelin fairly readily in the membrane of human spheroechinocytes I. Furthermore, this sphingomyelin-degrading activity is not affected by the presence of 5mM-Ca^{2+}, a powerful inhibitor of the sphingomyelinase C of *B. cereus* (Ikezawa *et al.*, 1978). It is possible that the substrate specificity of phospholipase C (*B. cereus*), as determined from the rates of hydrolysis of pure phospholipids in detergent micelles, does not necessarily reflect the substrate specificity shown towards a mixture of phospholipids in a particular biological membrane.

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References

Allan, D. & Michell, R. H. (1975) *Nature* (*London*) **258**, 348–349

Allan, D., Low, M. G., Finean, J. B. & Michell, R. H. (1975) *Biochim. Biophys. Acta* **413**, 309–316

Allan, D., Watts, R. & Michell, R. H. (1976) *Biochem. J.* **156**, 225–232
Allan, D., Thomas, P. & Michell, R. H. (1978) *Nature (London)* **276**, 289–290
Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
Bessis, M. (1972) *Nouv. Rev. Fr. Hematol.* **12**, 1–25
Bessis, M. & Weed, R. I. (1972) in *Scanning Electron Microscopy*, Part II, pp. 289–296, ITT Research Institute, Chicago
Birchmeier, W. & Singer, S. J. (1977) *J. Cell Biol.* **73**, 647–659
Feo, C. & Mohandas, N. (1977) *Nature (London)* **265**, 166–168
Graham, C., Avruch, J. & Fairbanks, G. (1976) *Biochem. Biophys. Res. Commun.* **72**, 701–708
Haradin, A. R., Weed, R. I. & Reed, C. F. (1969) *Transfusion* **9**, 229–237
Hough, E., Little, C. & Jynge, K. (1978) *J. Mol. Biol.* **121**, 567–570
Ikezawa, H., Mori, M., Ohyabu, T. & Taguchi, R. (1978) *Biochim. Biophys. Acta* **528**, 247–256
Laemmli, U. K. (1970) *Nature (London)* **277**, 680–685
Leavitt, R. D. (1971) *Nouv. Rev. Fr. Hematol.* **11**, 331–338
Lichtman, M. A., Marinetti, G. V. & Gordesky, S. E. (1974) *Nouv. Fr. Rev. Hematol.* **14**, 5–24
Little, C. & Otnaess, A.-B. (1975) *Biochim. Biophys. Acta* **391**, 326–333
Little, C. & Johansen, S. (1979) *Biochem. J.* **179**, 509–514
Little, C., Aurebekk, B. & Otnaess, A.-B. (1975) *FEBS Lett.* **52**, 175–179
Longster, G. H., Buckley, T., Sikorski, J. & Tovey, L. A. D. (1972) *Vox Sang.* **22**, 161–170
Nakao, M. (1974) in *Cellular and Molecular Biology of Erythrocytes* (Yoshikawa, H. & Rapoport, S. M., eds.), pp. 35–54, University Park Press, Baltimore
Nakao, M., Nakao, T., Tatinaba, M., Yoshikawa, H. & Abe, T. (1959) *Biochim. Biophys. Acta* **32**, 564–565
Otnaess, A.-B., Little, C., Sletten, L., Wallin, R., Johnsen, S., Flengrud, R. & Prydz, H. (1977) *Eur. J. Biochem.* **79**, 459–468
Roberts, M. F., Otnaess, A.-B., Kensil, C. A. & Dennis, E. A. (1978) *J. Biol. Chem.* **253**, 1252–1257
Roelofsen, B., Zwaal, R. F. A., Comfurius, P., Woodward, C. B. & Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* **241**, 925–929
Rumsby, M. G., Barnes, Y. M., Hoare, D. G. & Tovey, L. A. D. (1974) *Biochem. Soc. Trans.* **2**, 550–552
Rumsby, M. G., Trotter, J., Allan, D. & Michell, R. H. (1977) *Biochem. Soc. Trans.* **5**, 126–128
Rumsby, M. G., Sparrow, S. & Little, C. (1979) *Biochem. Soc. Trans.* **9**, 922–924
Shohet, S. B. & Greenquist, A. C. (1977) *Blood Cells* **3**, 115–131
Shukla, S. D., Billah, M. M., Coleman, R., Finean, J. B. & Michell, R. H. (1978*a*) *Biochim. Biophys. Acta* **509**, 48–57
Shukla, S. D., Coleman, R., Finean, J. B. & Michell, R. H. (1978*b*) *Biochim. Biophys. Acta* **512**, 341–349
Van Gastel, C., Van Den Berg, D., De Gier, J. & Van Deenen, L. L. M. (1965) *Br. J. Haematol.* **11**, 193–199
Weed, R. I., La Celle & Udkow, M. (1974) in *The Human Red Cell in vitro* (Greenwalt, T. J. & Jamieson, G. A., eds.), pp. 65–89, Grune and Stratton, New York
Westerman, M. P., Pierce, L. E. & Jensen, W. N. (1963) *J. Lab. Clin. Med.* **62**, 394–400
Wilbers, K. H., Haest, C. W. M., Von Bentheim, M. & Deuticke, B. (1979) *Biochim. Biophys. Acta* **554**, 388–399
Winterbourn, C. C. & Batt, R. D. (1970) *Biochim. Biophys. Acta* **202**, 1–8
Zwaal, R. F. A., Roelofsen, B., Comfurius, P. & Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* **233**, 474–479